A Role for Bioactivation and Covalent Binding within Epidermal Keratinocytes in Sulfonamide-Induced Cutaneous Drug Reactions[1]

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Cutaneous reactions are the most common manifestation of delayed-type hypersensitivity caused by sulfamethoxazole and dapsone. In light of the recognized metabolic and immunologic activity of the skin, we investigated the potential role of normal human epidermal keratinocytes in the development of these reactions. Adult and neonatal normal human epidermal keratinocytes metabolized sulfamethoxazole and dapsone to N-4-hydroxylamine and N-acetyl derivatives in a time-dependent manner. The latter was catalyzed by N-acetyltransferase 1 alone as normal human epidermal keratinocytes did not express mRNA for N-acetyltransferase 2. Investigation of metabolism-dependent toxicity of sulfamethoxazole and dapsone, and subsequent incubation of normal human epidermal keratinocytes with the respective hydroxylamine metabolites, demonstrated that these cells were resistant to the cytotoxic effects of sulfamethoxazole hydroxylamine but not dapsone hydroxylamine. With prior depletion of glutathione, however, normal human epidermal keratinocytes became susceptible to the toxicity of sulfamethoxazole hydroxylamine. Covalent adduct formation by sulfamethoxazole hydroxylamine was detected in normal human epidermal keratinocytes, even in the absence of cell death, and was increased with glutathione depletion. Major protein targets of sulfamethoxazole hydroxylamine were observed in the region of 160, 125, 95, and 57 kDa. Dapsone hydroxylamine also caused covalent adduct formation in normal human epidermal keratinocytes. Together, these observations provide a basis for our hypothesis that normal human epidermal keratinocytes are involved in the initiation and propagation of a cutaneous hypersensitivity response to these drugs. ***Key words: glutathione/hydroxylamine metabolites/hypersensitivity/sulfamethoxazole. J Invest Dermatol 114:1164–1173, 2000***

The use of trimethoprim, sulfamethoxazole (SMX) or dapsone (DDS) for opportunistic infections in human immunodeficiency virus (HIV) infected individuals is complicated by their association with a high incidence of delayed-type hypersensitivity (HS) reactions (Gordin *et al*, 1984; Medina *et al*, 1990). Because this increased frequency does not appear to be secondary to the effects of *Pneumocystis carinii* infection nor to the higher doses used in the treatment of immunodeficient individuals with *Pneumocystis carinii* pneumonia (Kovaks *et al*, 1984), HIV infection appears to be an important predisposing factor. Unfortunately, the pathogenesis of drug-induced HS reactions and the factors that contribute to their occurrence remain unknown.

Cutaneous reactions occurring after 7–14 d of therapy represent the most commonly observed clinical manifestation of many drug-induced HS reactions, including those caused by SMX and DDS. In many cases, relatively minor cutaneous effects are believed to precede more severe cutaneous eruptions (e.g., toxic epidermal necrolysis) or internal organ complications if drug therapy is not halted (Shear, 1990). Immunohistologic investigation of these cutaneous drug eruptions provides strong support for the hypothesized role of an immunologic component. Studies have documented significant lymphocytic infiltration, consisting primarily of dermal CD4+ and epidermal CD8+ T cells, into affected areas (Miyauchi *et al*, 1991; Villada *et al*, 1992; Hertl *et al*, 1993, 1995; Carr *et al*, 1994; Osawa *et al*, 1994; Hern *et al*, 1998). In one study, CD8+ dermal T cells from the lesional skin of a sulfonamide HS patient proliferated in response to SMX-modified liver microsomes (Hertl *et al*, 1995), suggesting a drug-specific immune response. Moreover, epidermal Langerhans cell depletion, believed to be a hallmark of a cutaneous HS response, has been

Manuscript received October 13, 1999; revised February 28, 2000; accepted for publication March 5, 2000.

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Abbreviations: BSO, buthionine sulfoximine; CDR, cutaneous drug reaction(s); DDS, dapsone; DDS-NOH, dapsone hydroxylamine; DEM, diethyl maleate; GSH, glutathione; HS, hypersensitivity; NAT, N-acetyltransferase; SMX, sulfamethoxazole; SMX-NOH, sulfamethoxazole hydroxylamine.

[1]Portions of this work were presented at the 38th Annual Meeting of the Society of Toxicology, New Orleans, LA, March 1999.

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noted in similar lesional skin sections (Villada *et al*, 1992; Carr *et al*, 1994).

Keratinocytes are the most abundant cell type in the skin and are considered to be a target of the immune-mediated damage during cutaneous reactions (Paul *et al*, 1996; Schnyder *et al*, 1998). Recent evidence, however, suggests that keratinocytes may actually play an active role in the initiation of these reactions due to their wide array of immunologic capabilities. Keratinocytes produce a large repertoire of cytokines (Stoof *et al*, 1994), including interleukin-1β and tumor necrosis factor α, which are critical initiators of Langerhans cell migration (Cumberbatch *et al*, 1997, 1999). They can also be induced to express a number of cell surface molecules such as intercellular adhesion molecule 1 (ICAM-1) and human leukocyte antigen (HLA) DR, both of which are upregulated in lesional skin (Shiohara *et al*, 1989; Miyauchi *et al*, 1991; Hertl *et al*, 1993, 1995; Osawa *et al*, 1994). Moreover, stressed keratinocytes appear to be able to activate dendritic epidermal T cells directly (Havran *et al*, 1991; Huber *et al*, 1995), though antigen-specific tolerance is observed in other situations (Nickoloff *et al*, 1986; Gaspari *et al*, 1988). These data suggest that keratinocytes may be critical in the elicitation, propagation, as well as termination of a cutaneous immune response to drugs and other foreign stimuli.

Interestingly, skin cells, particularly keratinocytes, have also become increasingly recognized for their metabolic capabilities (Kao and Carver, 1990; Merk *et al*, 1996). This is of particular relevance for drug-induced cutaneous reactions as it is unlikely that reactive metabolites formed in the liver would "survive" transit to distant cutaneous sites. If formation of reactive species is indeed a necessary initiating event in drug-induced HS, local (i.e., cutaneous) bioactivation might be expected to be more important than hepatic formation of such metabolites.

Based upon these data, we propose a new model for the development of cutaneous drug reactions (CDRs) to sulfonamides. This model hypothesizes that primary or secondary metabolism of SMX (or a metabolite) by epidermal keratinocytes creates reactive hydroxylamine metabolites (SMX-NOH) in the microenvironment of the skin. SMX-NOH, generated from local as well as systemic sources, might then cause a direct cytotoxic or an indirect immuno-modulatory effect that promotes the development of a CDR. Studies described herein represent our initial characterization of the potential role of epidermal keratinocytes in sulfonamide-induced HS reactions.

MATERIALS AND METHODS

Materials Normal human epidermal keratinocytes and keratinocyte culture media were obtained from Clonetics (BioWhittaker, San Diego, CA). Hydroxylamine metabolites of DDS and SMX were synthesized as described previously (Rieder *et al*, 1988; Vage *et al*, 1994) and determined by high performance liquid chromatography (HPLC) to be more than 97% pure. Drugs and chemical reagents, including those used in electrophoresis, were obtained from Sigma (St. Louis, MO). Ninety-six-well tissue-culture-treated and enzyme-linked immunosorbent assay (ELISA) treated polystyrene microtiter plates were obtained from Rainin Instruments (Woburn, MA). The fluorescent nucleic acid dye YO-PRO-1 iodide was obtained from Molecular Probes (Eugene, OR). Immunochemicals were purchased from Amersham Pharmacia Biotech (Piscataway, NJ). Solvents and buffer salts were purchased from Fisher Scientific (Fair Lawn, NJ) or Sigma.

Cell culture Both neonatal and adult normal human epidermal keratinocytes were obtained as cryopreserved 1st passage cells from Clonetics. Normal human epidermal keratinocytes were grown in T-75 cm^2 flasks using basal media (KBM-2; 0.15 mM Ca^{2+}) supplemented with bovine pituitary extract (7.5 mg per ml), human epidermal growth factor (0.1 ng per ml), insulin (5 μg per ml), hydrocortisone (0.5 μg per ml), epinephrine, transferrin, gentamicin (50 μg per ml), and amphotericin (50 ng per ml) (KGM-2; Clonetics) at 37°C/5% CO_2. Media was replaced every 2–3 d. When near confluence (70%–90%), normal human epidermal keratinocytes were disaggregated using 0.025% trypsin/0.01% ethylenediamine tetraacetic acid in HEPES and either subcultured for experimental use or cryopreserved in liquid nitrogen for future purposes. All experimental procedures were performed using 2nd to 4th passage cells.

***In vitro* metabolism** Normal human epidermal keratinocytes were grown until approximately 50% confluent in KGM-2 (with low Ca^{2+}, 0.15 mM). Media was then replaced with high Ca^{2+} KGM-2 (1.25-2 mM) for 24–48 h, whereupon cells were collected for experimental use. Normal human epidermal keratinocyte suspensions (7×10^5 cells per ml) in KBM-2 with high Ca^{2+} (1.25-2 mM) and containing 1–2 mM ascorbic acid were then distributed to glass capped tubes and allowed to recover for several hours. DDS or SMX (1 mM final concentration) were added in dimethylsulfoxide (0.25% vol/vol) and incubations were performed at 37°C. Throughout a 24 h incubation, reactions were halted by adding 5 ml ethyl acetate, samples were extracted, and metabolites were resolved from their parent arylamines by HPLC. Separation was performed using a previously described assay protocol (Coleman *et al*, 1989). N-hydroxy and N-acetyl metabolites of DDS and SMX were quantified based upon externally generated standard curves.

Detection of N-acetyltransferase (NAT) mRNA RNA was isolated from adult and neonatal normal human epidermal keratinocyte cell pellets using the RNeasy Kit (Qiagen). Analysis of human placental tissue, known to express both NAT1 and NAT2, was done simultaneously as a positive control. Reverse transcription of RNA was performed using the Advantage RT Kit following the manufacturer's instructions (Clontech; Palo Alto, CA). Polymerase chain reaction (PCR) amplification of the reverse transcribed RNA was carried out using primers (5′-TTCACCTTCTCC-TGCAGGTG-3′ and 5′-TCTGCGGTCTGCAAGGAAC-3′) designed to amplify both NAT1 and NAT2. PCR products were subsequently digested with the restriction enzyme *Alw*NI and samples were run on a 2% agarose gel. *Alw*NI will cleave NAT1 but not NAT2, yielding two separate bands only when both NAT1 and NAT2 are present.

***In vitro* cytotoxicity** Initially, parent arylamine (DDS and SMX) toxicity was determined using normal human epidermal keratinocytes that were previously cultured in high Ca^{2+} media (1.25 mM Ca^{2+}) for 12–24 h. Cells were subcultured in 24 well microtiter plates (KGM-2 with high Ca^{2+}) and allowed to recover and attach overnight. Cell death was determined at various time points over 72 h, in the continuous presence of 1 mM DDS or SMX and high Ca^{2+} (in KBM-2), using the membrane-impermeable, nucleic-acid-binding fluorescent dye YO-PRO-1 as described previously (Tabatabaei *et al*, 1997; Reilly *et al*, 1999).

In other experiments, the effects of synthetic hydroxylamine metabolites (DDS-NOH and SMX-NOH) on normal human epidermal keratinocyte viability were determined. Normal human epidermal keratinocytes were subcultured in 96 well microtiter plates (~5.0×10^4 cells per 200 μl per well) and cells were allowed to recover and attach overnight (~12–18 h). The medium was then replaced with KBM-2 (0.15 mM Ca^{2+}) and hydroxylamines were added in dimethylsulfoxide (1% vol/vol). After a further 3 h incubation, the medium was replaced with either KBM-2 or KGM-2, both containing 4 μM YO-PRO-1, and fluorescence was monitored over 16 h as described previously (Tabatabaei *et al*, 1997; Reilly *et al*, 1999). Cytotoxicity results were confirmed using the standard MTT assay with minor modifications (Reilly *et al*, 1998).

Arylamine distribution into normal human epidermal keratinocytes Entry of the parent arylamines DDS and SMX into normal human epidermal keratinocytes was used as an indirect indicator of metabolite flux across cell membranes. Normal human epidermal keratinocytes, cultured in KGM-2, were incubated with 200 μM SMX or DDS, and samples were collected over 2 h. After centrifugation, the extracellular medium and cell pellet (with some residual medium) were separated, extracted with ethyl acetate, and then analyzed by HPLC for parent arylamine concentrations as described previously (Coleman *et al*, 1989).

Effect of glutathione (GSH) depletion The effect of hydroxylamines on cellular GSH status was determined by incubating adult normal human epidermal keratinocytes with 0.1 and 1 mM DDS-NOH or SMX-NOH at 37°C/5% CO_2. After 1 or 6 h, 70% perchloric acid was added to halt further reaction and samples were frozen at –80°C until analysis. Similarly, the effect of the GSH depleting agents, diethyl maleate (DEM) and buthionine sulfoximine (BSO), on normal human epidermal keratinocyte GSH levels was determined by adding various concentrations to normal human epidermal keratinocytes for 1 or 24 h, respectively, and samples were stored as described above. GSH content was determined using an ion-exchange HPLC assay described previously (Fariss and Reed, 1987).

The effect of GSH depletion on hydroxylamine-induced toxicity was subsequently determined by pretreating normal human epidermal keratinocytes *in vitro* with various concentrations of DEM or BSO. After 1 h (DEM) or 24 h (BSO), normal human epidermal keratinocytes were exposed to

increasing concentrations of DDS-NOH or SMX-NOH for 3 h and cell death was determined over the following 16 h using YO-PRO-1 as described above.

Covalent adduct formation In order to test the hypothesis that *in vitro* cytotoxicity is secondary to covalent binding of reactive metabolites to critical cellular macromolecules, normal human epidermal keratinocytes (~5×10^5 cells per ml), with and without prior GSH depletion, were incubated with increasing concentrations of SMX-NOH (0.1-2 mM) or 2 mM SMX for 3–24 h at 37°C. Cell pellets were then washed extensively with 20 ml ice-cold phosphate-buffered saline (PBS, pH 7.4), each time centrifuging at 1500*g* to pellet all viable and nonviable cells. Final cell pellets were lyzed with deionized water, using ultrasonication and freezing to ensure complete lysis. The protein content of each sample was then determined using the Bio-Rad protein assay kit (Bio-Rad Laboratories), with bovine serum albumin as a standard.

For comparison and to provide further information on the relationship between covalent adduct formation and cytotoxicity, similar incubations were also carried out using peripheral blood mononuclear cells (PBMC) from a normal human volunteer. PBMC were separated from whole human blood using Histopaque-1077 (Sigma, St Louis, MO) as described previously (Reilly *et al*, 1999), and incubations with SMX-NOH or SMX were performed in Hanks' balanced salt solution for 3 h at 37°C. PBMC pellets were then washed extensively with ice-cold PBS and lyzed with deionized water/ultrasonication/freezing; protein content was determined as described above.

Similar incubations of normal human epidermal keratinocytes and PBMC using DDS-NOH (100 μM) and DDS (1 mM) were also performed as described above.

ELISA ELISA analysis for detection of covalent adducts was performed as described previously (Kenna *et al*, 1984). Briefly, PBMC or normal human epidermal keratinocyte cell homogenate, P9 (mitochondrial, plasma, and nuclear membranes), and S9 (cytosol and microsomes) fractions were diluted to 50 μg per ml, and 100 μl was adsorbed onto polystyrene microtiter plates (Rainin) at 4°C for 16 h. Wells were washed three times with casein-tri(hydroxymethyl)-aminomethane (Tris) buffer (0.5% casein, 0.9% NaCl, 0.01% Thimerosal, 10 mM Tris-HCl, pH 7.6) and then blocked with casein-Tris at room temperature for 1 h. After an additional wash, wells were incubated for 2 h at 4°C with 100 μl of an anti-SMX rabbit serum characterized previously (Cribb *et al*, 1996b) (provided by Dr. Alastair E. Cribb, Department of Anatomy and Physiology, Atlantic Veterinary College, PEI, Canada) that had been preabsorbed against the nonspecific protein keyhole limpet hemocyanin (KLH) (Kenna *et al*, 1984) and then diluted in PBS (1:1000). The specificity of antibody binding was assessed by preincubating antisera with SMX or DDS for 1 h prior to adding to sample wells. Rabbit antisera was also raised against a DDS-KLH conjugate prepared using the Imject Immunogen EDC Kit (Pierce) and utilized for DDS-NOH- and DDS-treated samples at a dilution of 1:1000. All dilutions of antisera were based upon concentration-response curves generated using solid phase drug antigens and were selected from the saturating point of each curve such that adduct levels would be the limiting portion of assay reactions. Wells were subsequently washed four times with casein-Tris buffer and incubated for 2 h with alkaline-phosphatase-conjugated goat anti-rabbit secondary antibodies (1:1000 in casein-Tris; Amersham) at room temperature. After washing four times with casein-Tris and twice with PBS, antibody binding was detected using the colorimetric substrate p-nitrophenylphosphate (1 mg per ml, final concentration). After 0.5 h incubation, reactions were halted with 3 M NaOH and optical density was determined at 405 nm using a Vmax kinetic microplate reader (Molecular Devices). Dot blots using a secondary antirabbit horseradish-peroxidase-conjugated antibody with chemiluminescent detection were utilized as a potentially more sensitive method to confirm ELISA results.

Immunoblots Proteins (75 μg per lane) were resolved overnight at 4°C using 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) full-size gels. Because addition of reducing agents to the loading buffer was expected to eliminate covalent binding (Cribb *et al*, 1996b), all gels were run using nonreducing conditions (i.e., no dithiothreitol or β-mercaptoethanol). Electrophoretic transfer of proteins to nitrocellulose membranes was performed in transfer buffer (25 mM Tris, pH 8.3, 192 mM glycine, 0.1% SDS) containing 20% methanol for 2 h at 4°C according to standard procedures, whereupon membranes were stored at –20°C until immunodetection.

Membranes were moistened with deionized water, washed briefly with PBS containing 5% nonfat dry milk and 2% bovine serum albumin (blocking buffer), and then blocked for 1 h at room temperature in blocking buffer. Anti-SMX rabbit serum (or anti-DDS rabbit serum where appropriate) was then added (1:1000 dilution) in blocking buffer containing 0.1% Tween 20 and incubations were continued for 2 h at room temperature with mild shaking. Subsequently, the membranes were rinsed with PBS/0.1% Tween 20 and washed for an additional 30 min with three changes of PBS/Tween. Anti-rabbit horseradish-peroxidase-conjugated secondary antibody (from sheep; Amersham) was then added in blocking buffer (1:2000 dilution) and incubated for 2 h at room temperature with agitation. After washing as described above, immunoreactions were visualized using an enhanced chemiluminescence system (Amersham) wherein membranes were exposed to 13 × 18 cm autoradiographic film (Biomax MR, Kodak) for enough time to obtain suitable exposure (1 s to 5 min). Longer exposures were also performed to test for low level immunoreactivity of negative lanes.

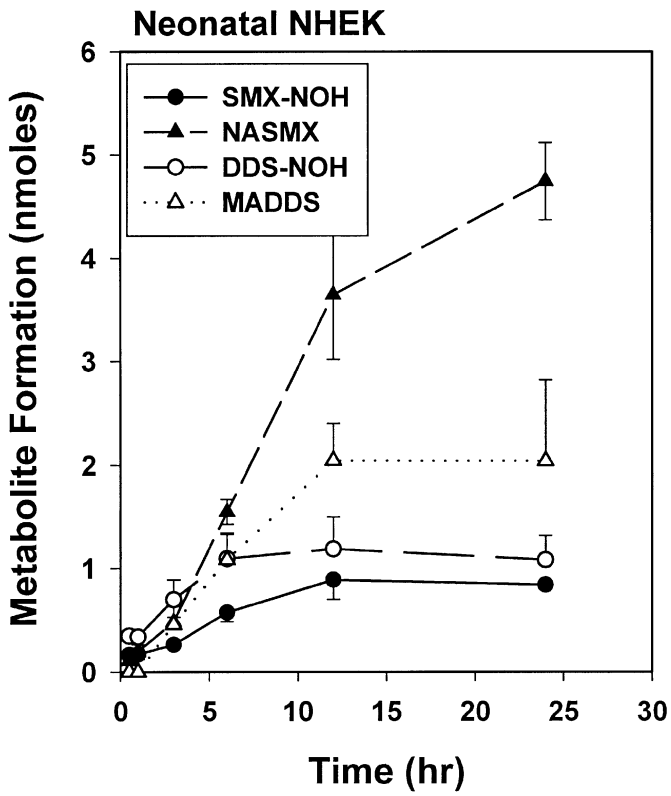

Figure 1. Biotransformation of SMX and DDS to N-hydroxy and N-acetyl metabolites by normal human epidermal keratinocytes (NHEK). Neonatal normal human epidermal keratinocytes were incubated with 1 mM SMX or DDS in basal media with high Ca^{2+} and 2 mM ascorbic acid and reactions were halted at various time points over 24 h. Metabolites were extracted from incubation media, separated by HPLC, and quantified based upon externally generated standard curves. Results are presented as the mean (SD) of triplicate samples. Comparable results were observed using adult normal human epidermal keratinocytes.

RESULTS

Metabolic activity of normal human epidermal keratinocytes Neonatal normal human epidermal keratinocytes incubated with 1 mM SMX or DDS in high Ca^{2+} media were capable of metabolizing both arylamines to N-4-hydroxy (i.e., hydroxylamine) and N-acetyl metabolites (**Fig 1**). Greater amounts of N-acetyl SMX were formed than monoacetyl DDS. Adult normal human epidermal keratinocytes showed a very comparable metabolic profile, although accurate quantitation of hydroxylamine formation was hampered by the presence of interfering peaks in the media (data not shown). All metabolites were formed in a time-dependent manner from 0.5 to 24 h in both neonatal and adult normal human epidermal keratinocyte cultures and were dependent upon the presence of viable normal human epidermal keratinocytes. In order to investigate which NAT(s) might be responsible for the observed acetylation activity, reverse

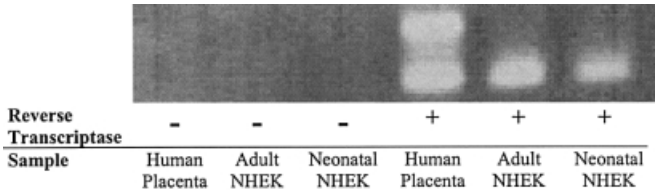

Figure 2. Reverse transcriptase PCR analysis of normal human epidermal keratinocyte mRNA for detection of *N*-acetyltransferase isoforms. mRNA isolated from normal human epidermal keratinocytes was reverse transcribed and then amplified by PCR using oligonucleotides designed to amplify both NAT1 and NAT2. Human placental tissue, known to express both NAT1 and NAT2, was analyzed simultaneously as a positive control. Elimination of reverse transcriptase enzyme served as a negative control for each sample. PCR products were digested with *Alu*NI, which cuts NAT1 and not NAT2, and resolved on 2% agarose gels.

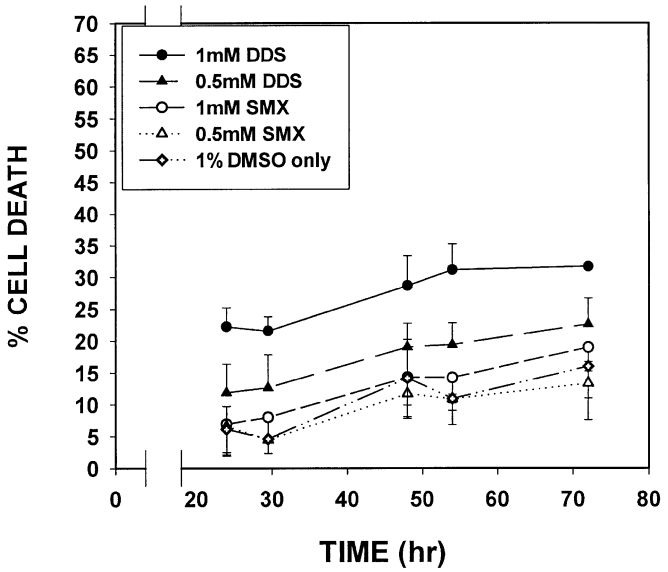

Figure 3. Time-dependent parent-arylamine-induced normal human epidermal keratinocyte cell death. DDS or SMX was added to microtiter plate wells containing confluent normal human epidermal keratinocytes bathed in high Ca^{2+} basal media with the membrane-impermeable, nucleic-acid-binding fluorescent dye YO-PRO-1. Fluorescence was monitored over 72 h as an indication of the loss of membrane integrity and resultant cell death. Results shown are the mean (SD) of triplicate wells.

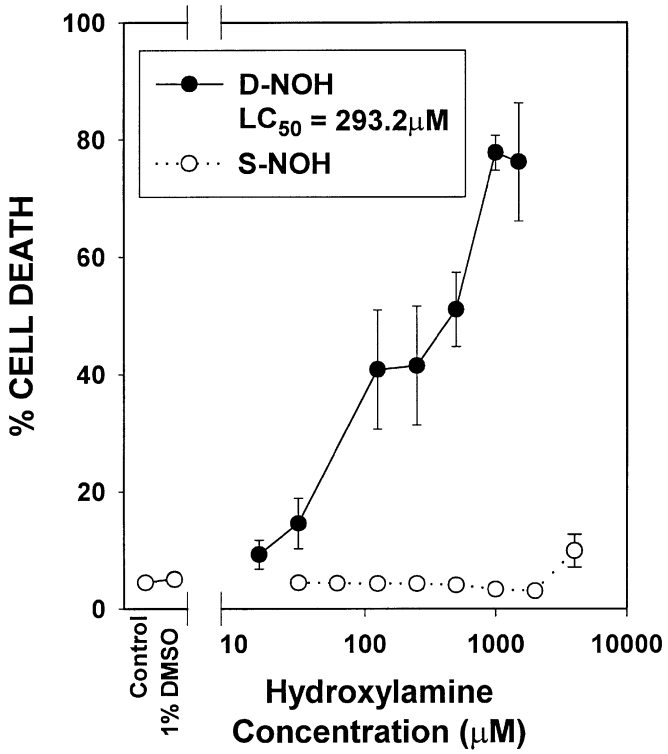

Figure 4. Hydroxylamine-induced normal human epidermal keratinocyte cell death. Normal human epidermal keratinocytes in basal media were incubated with increasing concentrations of DDS-NOH or SMX-NOH for 3 h at 37°C. The medium was then replaced with fresh medium containing the membrane-impermeable, nucleic-acid-binding fluorescent dye YO-PRO-1 and fluorescence was determined over the following 16 h. Results shown are the percentage mean (SD) cell death values of quadruplicate samples determined 16 h after hydroxylamine exposure. The LC_{50} for DDS-NOH-induced toxicity was determined using linear regression analysis of cell death values from 20% to 80%.

transcriptase PCR analysis of normal human epidermal keratinocyte mRNA was performed. As shown in **Fig 2**, both neonatal and adult normal human epidermal keratinocytes appear to express message for NAT1 but not NAT2.

Normal human epidermal keratinocyte cytotoxicity Detection of reactive metabolite formation by normal human epidermal keratinocytes suggested that incubation with SMX and DDS might also cause metabolism-dependent toxicity. Initial assessments of normal human epidermal keratinocyte cell death caused by SMX or DDS indicated, however, that SMX alone did not induce a toxic response above control levels, regardless of concentration or length of incubation time (**Fig 3**). In contrast, DDS caused time-dependent normal human epidermal keratinocyte cell death over 72 h (**Fig 3**). DDS-induced toxicity also occurred in a concentration-dependent manner after a 24 h incubation (data not shown).

Investigation of this discrepancy using synthetic hydroxylamine metabolites indicated a marked difference in the susceptibility of normal human epidermal keratinocytes to SMX-NOH and DDS-NOH. Whereas normal human epidermal keratinocytes demonstrated a concentration-dependent susceptibility to DDS-NOH-induced toxicity, they appeared to be virtually resistant to the cytotoxic effects of SMX-NOH (**Fig 4**). DDS-NOH-induced toxicity increased over time and similar toxicity profiles were observed for each metabolite, irrespective of the use of basal or growth media during incubations (data not shown). Investigation of the potential contribution of dispositional differences to these observations indicated that partitioning of SMX and DDS into normal human epidermal keratinocytes does not appear to play a role (data not shown).

Interestingly, differences were observed upon evaluation of the effect of hydroxylamine metabolites on normal human epidermal keratinocyte intracellular GSH content. At 1 and 6 h, SMX-NOH caused a substantial reduction in GSH levels (**Fig 5**) despite the peculiar absence of a cytotoxic effect. For instance, at 0.1 mM SMX-NOH, GSH was depleted to about 45% of control levels after 6 h. Whereas DDS-NOH also caused GSH depletion, it appeared to be less pronounced and shorter-lived than the depletion caused by incubation with SMX-NOH. Furthermore, concentrations of DDS-NOH that caused significant normal human epidermal keratinocyte cell death caused minimal or no GSH depletion. These data suggested that GSH may be particularly important in the detoxification of SMX-NOH. To test this hypothesis, the effect of GSH depletion on hydroxylamine-induced cytotoxicity was evaluated. Both DEM and BSO, each known to deplete GSH but by different mechanisms, caused significant depletion of normal human epidermal keratinocyte GSH content (data not shown). Incubation with 0.4 mM DEM or 0.5 mM BSO, concentrations found to cause about 50% GSH depletion (data not shown), caused minimal or no toxicity alone (**Fig 6**). In each case,

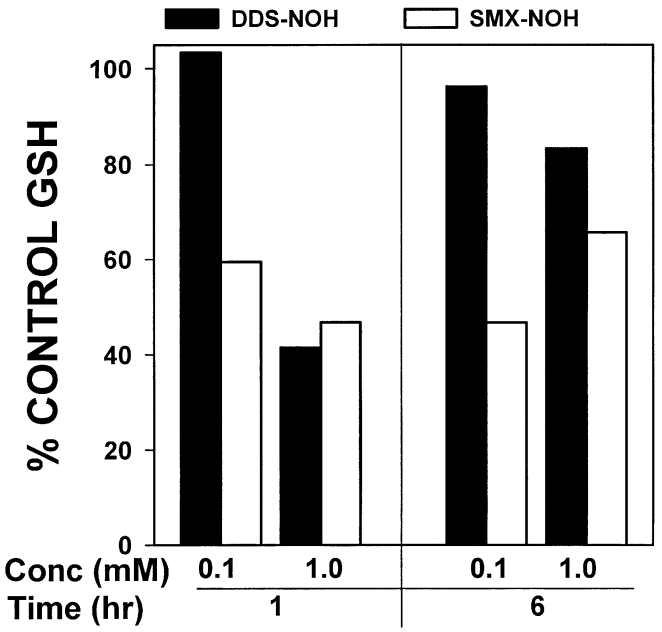

Figure 5. Depletion of normal human epidermal keratinocyte GSH content by hydroxylamine metabolites. Adult normal human epidermal keratinocytes were incubated with SMX-NOH or DDS-NOH for 1 or 6 h. Reactions were terminated with 70% perchloric acid and GSH content was determined by HPLC analysis. Results are presented as the percentage of control GSH levels at 1 h (24.34 nmol per 10^6 cells) and 6 h (28.58 nmol per 10^6 cells), respectively, and represent the average of duplicate samples for each condition.

however, the toxic effect of hydroxylamine metabolites was potentiated by prior GSH depletion (**Fig 6**). Importantly, prior depletion of GSH made normal human epidermal keratinocytes susceptible to SMX-NOH-induced toxicity at concentrations where no significant toxicity was observed in the absence of this pretreatment. Time-course experiments also revealed enhanced toxicity of each hydroxylamine at earlier time points (data not shown).

Covalent binding Reactive metabolites of SMX and DDS might also form covalent adducts with cellular macromolecules and subsequently act as neoantigens against which an immune response may be initiated. In order to test this hypothesis, normal human epidermal keratinocytes were incubated with SMX, SMX-NOH, DDS, or DDS-NOH and covalent adduct formation was probed using rabbit antisera raised against SMX-KLH or DDS-KLH conjugates. Additional incubations were also performed with PBMC, a cell type that has previously been shown to be susceptible to hydroxylamine-induced toxicity (Reilly *et al*, 1998; 1999). As demonstrated by the ELISA analysis shown in **Figs 7** and **10**, both SMX-NOH and DDS-NOH appear to form covalent adducts with proteins in normal human epidermal keratinocytes and PBMC, even at relatively low concentrations that are predicted to be achieved *in vivo*. Analysis of the S9 (cytosol and microsomes) and P9 (mitochondrial, plasma, and nuclear membranes) cell fractions of SMX-NOH incubations showed comparable levels of adduct (data not shown). Incubation of cells with SMX or DDS did not yield detectable adduct formation under any circumstances, although this finding is presumably the result of adduct levels being below detectable limits due to the small amount of hydroxylamine formed rather than an absence of formation. Cribb *et al* (1996a,b) observed similar limitations in their attempt to detect SMX adducts in an *in vivo* system. Moreover, the specificity of SMX- or DDS-protein adducts was confirmed by the failure to detect a positive response against native cellular proteins and the ability to eliminate antibody binding by competitive inhibition with SMX and DDS (**Fig 8**). In each case, some cross-reactivity was observed in

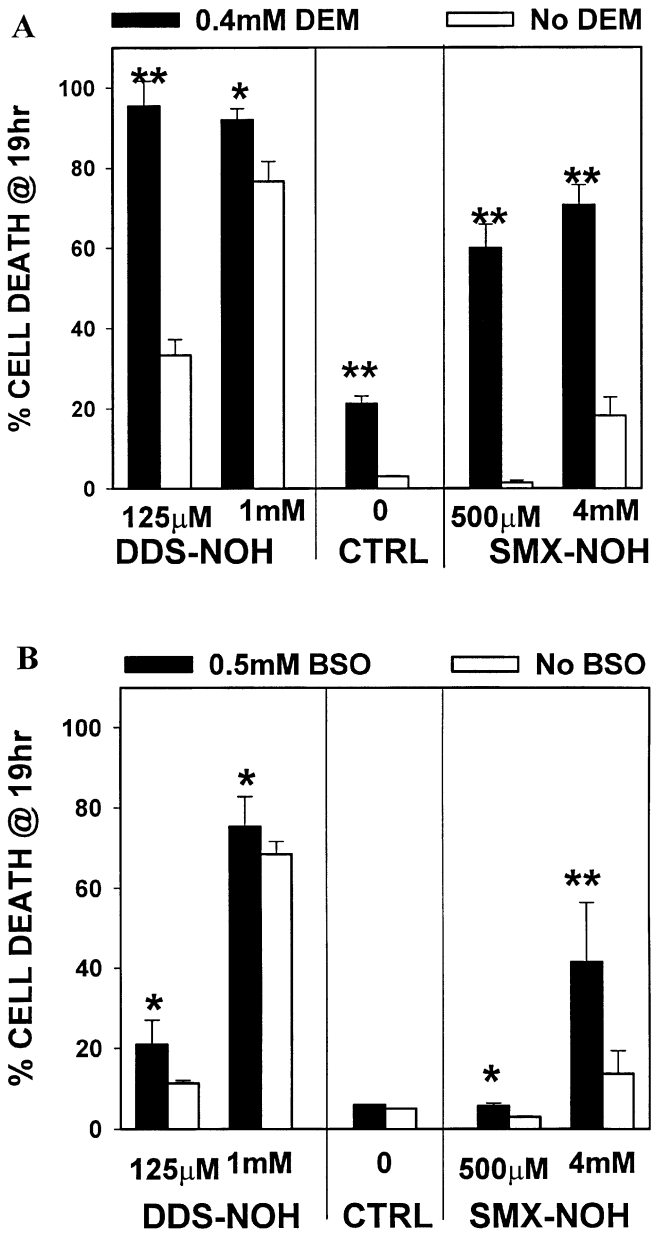

Figure 6. Potentiation of hydroxylamine-induced normal human epidermal keratinocyte cell death by GSH depletion. Adult normal human epidermal keratinocytes were incubated for 1 h with 0.4 mM DEM (*A*) or 24 h with 0.5 mM BSO (*B*) before 3 h exposure to hydroxylamine metabolites. Results represent the percentage cell death 16 h after hydroxylamine exposure and are the mean (SD) of quadruplicate samples. Similar results were also achieved upon repeat analysis using different concentrations of GSH depleting agents. *$p < 0.05$; **$p < 0.005$.

competitive inhibition analysis using SMX and DDS. Importantly, SMX-NOH formed covalent adducts in normal human epidermal keratinocyte incubations at concentrations well below those that induced cell death, a response that was further augmented by depletion of GSH levels with DEM prior to incubation with SMX-NOH (**Fig 7**). These results were substantiated using dot blot analysis with chemiluminescent detection (data not shown).

Subsequent resolution of these proteins by SDS-PAGE demonstrated that major SMX-protein adducts were formed in the region of 160, 124, 95, and 57 kDa (**Fig 9**). Additional faint bands were observed at approximately 146, 90, 70–80 (three bands), 45, and 25–40 kDa (four bands). Although SMX-NOH appeared to show a

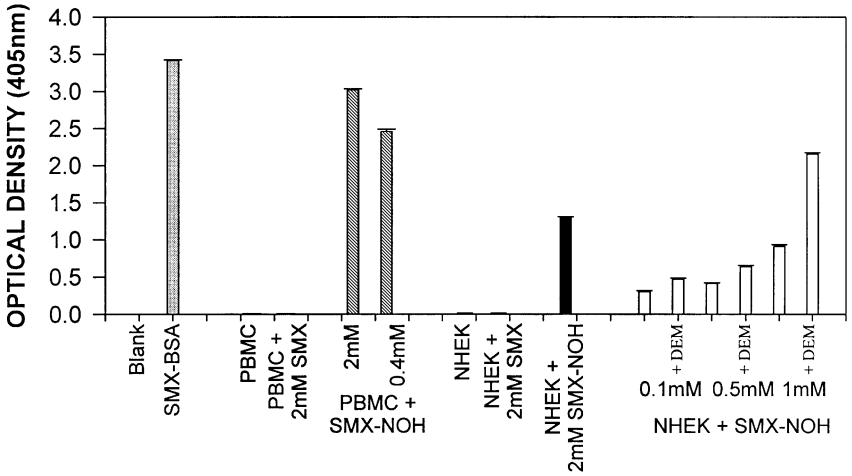

Figure 7. Detection of SMX-protein adducts by ELISA. PBMC and normal human epidermal keratinocytes (with or without prior GSH depletion) were incubated for 3 h with SMX-NOH, SMX, or vehicle alone and then washed extensively before cell lysis. Cell homogenates were coated onto microplate wells (5 μg protein per well) and adducts were detected using rabbit antisera (1:1000 dilution) raised against an SMX-KLH conjugate. Results are the mean (SD) of quadruplicate determinations and are representative of several repeat experiments.

nearly identical binding pattern in PBMC and normal human epidermal keratinocytes, there did appear to be a 39 kDa band in normal human epidermal keratinocytes that was not present in PBMC. It is unclear whether this observation is due to the differential expression of proteins in these cell types. Importantly, adducted proteins in normal human epidermal keratinocytes were detected in the absence of cell death and were increased by prior GSH depletion.

Preliminary experiments with DDS-NOH indicated that covalent adduct formation also occurs (**Fig 10**), although apparent targets were more diffuse over a much wider range of molecular masses (data not shown). These data suggest the possibility of nonspecific binding of DDS-NOH to many accessible molecules as a result of its high reactivity or the proteolytic breakdown of larger protein targets to smaller substituents.

DISCUSSION

Bioactivation to a reactive hydroxylamine metabolite (SMX-NOH and DDS-NOH) appears to be a critical initiating step in sulfonamide- and sulfone-induced HS reactions (Pohl *et al*, 1988; Uetrecht, 1992; Cribb *et al*, 1996a; Park *et al*, 1998). The sequence of events linking SMX-NOH or DDS-NOH to the development of idiosyncratic toxicity and the factors that promote such a response remain unknown (Cribb *et al*, 1996b). A large part of the problem stems from our inability to reproduce drug-induced HS events in a valid *in vitro* or *in vivo* model system. Moreover, it is difficult to equate the systemic formation of reactive metabolites of many drugs, including sulfonamides (e.g., SMX) and sulfones (e.g., DDS), with the cutaneous manifestations commonly observed in patients. Because epidermal keratinocytes are now recognized for their metabolic (Merk *et al*, 1996) and immunologic (Nickoloff, 1991) capabilities, we have undertaken a series of experiments aimed at investigating the potential role of normal human epidermal keratinocytes in HS-type cutaneous reactions to sulfonamides. In contrast to drugs such as halothane, tienilic acid, dihydralazine, diclofenac, and vesnarinone, where bioactivation, covalent binding, and manifestations of toxicity all seem to correspond (Uetrecht, 1999), hepatic bioactivation of SMX and DDS to reactive hydroxylamine metabolites does not correspond well with the development of a cutaneous HS response. Others have proposed that extrahepatic bioactivation by myeloperoxidase or prostaglandin H-synthase (PGHS; also known as cyclooxygenase or COX) in phagocytic cells may have particular toxicologic relevance (Uetrecht *et al*, 1988, 1993; Cribb *et al*, 1990). Here, we have made the novel observation that normal human epidermal

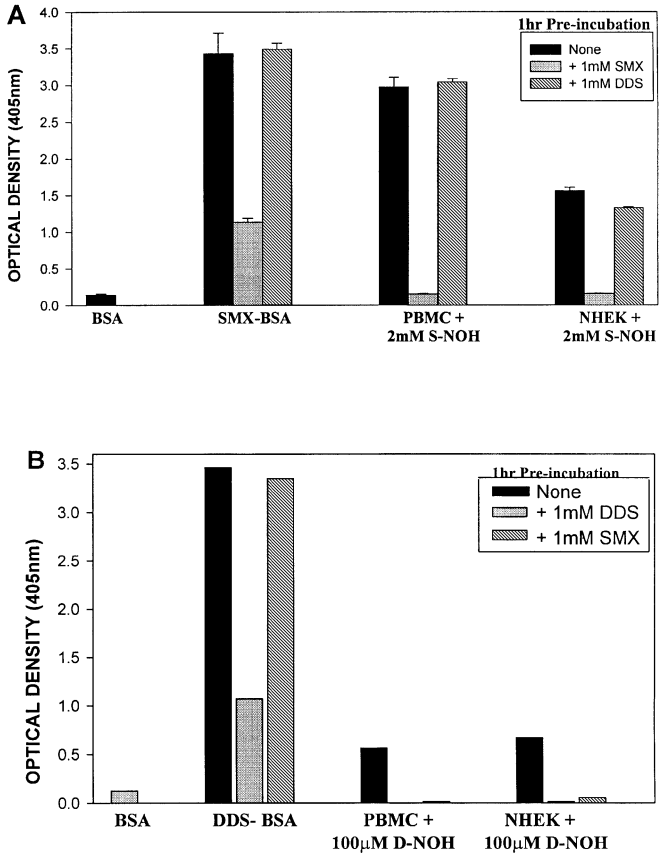

Figure 8. Competitive inhibition of antibody binding to drug-protein adducts. Rabbit antisera (1:1000) raised against (*A*) SMX-KLH or (*B*) DDS-KLH conjugates were preincubated with 1 mM SMX or DDS for 1 h before subsequent ELISA analysis (see legend to **Fig 7**). Results are the mean of quadruplicate determinations and are representative of individual experiments.

keratinocytes also appear capable of bioactivating arylamines to reactive hydroxylamine species, although further study is needed to elucidate the enzyme(s) presumed to be mediating this process. One might argue that the hydroxylamine levels observed are insufficient to elicit a significant response. Given the preponderance

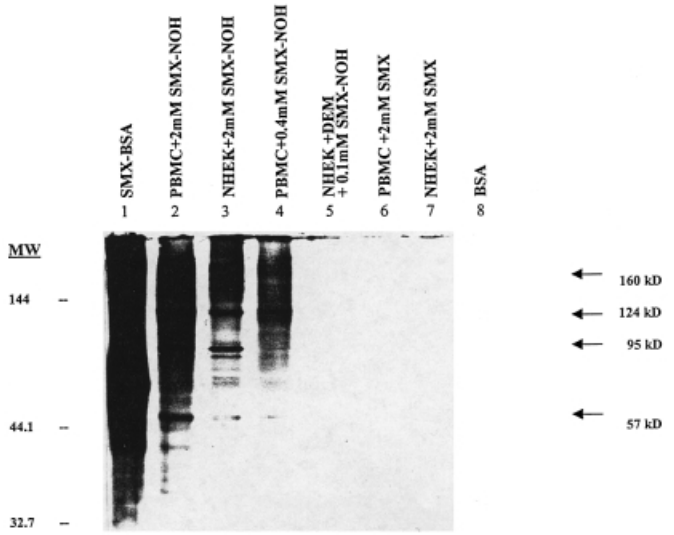

Figure 9. Immunoblot of SMX-protein adducts. Homogenates (75 μg per lane) were resolved by SDS-PAGE on 10% gels, transferred to nitrocellulose, and probed with anti-SMX serum (1:1000 dilution). The migration patterns of marker proteins are shown on the left and the apparent molecular weights of major protein adducts are identified on the right.

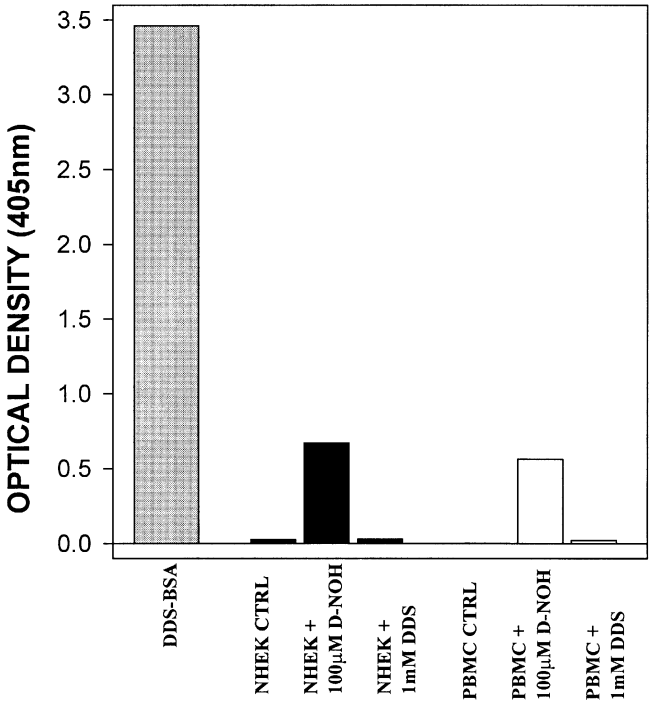

Figure 10. Detection of DDS-protein adducts. PBMC and normal human epidermal keratinocytes were incubated for 3 h with DDS-NOH, DDS, or vehicle alone and then washed extensively before cell lysis. Cell homogenates were coated onto microplate wells (5 μg protein per well) and adducts were detected by ELISA using rabbit antisera (1:1000 dilution) raised against a DDS-KLH conjugate. Results are the mean of quadruplicate determinations.

of keratinocytes in the skin, however, the unknown dose–response relationship that promotes the development of HS reactions, and the possibility that bioactivation may be inducible under a variety of conditions (including viral infections), keratinocyte-mediated hydroxylamine formation within the microenvironment of the skin may have toxicologic implications.

Interestingly, normal human epidermal keratinocytes were also found to possess NAT activity, apparently through NAT1 alone. Because N-acetylation is believed to serve a detoxification function, it is noteworthy that keratinocytes only appear to express NAT1, a finding that, to our knowledge, has not been reported previously. Whereas N-acetylation of SMX is catalyzed primarily by NAT1 (Cribb *et al*, 1993), recent data indicate that polymorphic NAT2 catalyzes the acetylation of SMX-NOH to an acetoxy ester (Nakamura *et al*, 1995) that is nontoxic (Nuss *et al*, 1996) and unable to covalently bind cellular macromolecules (Cribb *et al*, 1996b). This lack of NAT2 expression within normal human epidermal keratinocytes therefore suggests that they may be less able to detoxify SMX-NOH molecules once formed. Moreover, a decrease in cutaneous N-acetylation activity, as was recently reported in patients with severe CDRs,[3] may also decrease cutaneous clearance of the parent drug SMX, allowing more to be metabolized via oxidative pathways.

We subsequently hypothesized that modulation of biochemical and/or molecular events within normal human epidermal keratinocytes by reactive hydroxylamine species may play a critical role in the development of HS reactions. Unlike DDS, SMX was unable to cause metabolism-dependent normal human epidermal keratinocyte cell death regardless of concentration or incubation time. These results could not be attributed to distributional differences. Instead, we observed significant differences in the effects of SMX-NOH and DDS-NOH on normal human epidermal keratinocytes. Whereas DDS-NOH caused significant cytotoxicity in the presence of minimal or no GSH depletion (~40% cell death but < 4% GSH depletion at 0.1 mM DDS-NOH), SMX-NOH substantially depleted GSH levels in the absence of a cytotoxic effect. In fact, normal human epidermal keratinocytes appeared to be resistant to the toxicity of SMX-NOH unless GSH levels were depleted, whereupon normal human epidermal keratinocytes became susceptible to its cytotoxic effects. In combination with the significant GSH depletion caused by SMX-NOH (~40%–50% at 100 μM), these data suggest that GSH is important for the prevention of SMX-NOH-induced normal human epidermal keratinocyte toxicity, probably through its ability to prevent auto-oxidation to the ultimate toxin, nitroso-SMX (Cribb *et al*, 1991). Alternatively, resistance to SMX-NOH-induced toxicity might be due to increased amount of reduced nicotinamide adenine dinucleotide phosphate dependent reduction of SMX-NOH to SMX (Cribb *et al*, 1995), which has previously been shown to reduce covalent binding of SMX-NOH (Cribb *et al*, 1996b). In light of the marked effect of GSH depletion, however, this seems to be an unlikely explanation. Interestingly, these data contrast with a previous study (Cribb *et al*, 1991) wherein significant depletion of GSH levels within mononuclear leukocytes was only observed at higher concentrations of SMX-NOH (>300 μM) and was not necessary for *in vitro* cytotoxicity. It is currently unclear how these contrasting observations with SMX-NOH relate, nor is it known if the apparent mechanistic differences noted between SMX-NOH and DDS-NOH translate into the clinically observed differences in the frequency of HS reactions. The fact that normal human epidermal keratinocytes are present at the site of reaction, however, suggests that this cell type is probably a more relevant model system for further study.

The observations presented here with SMX-NOH, which place significant weight on the detoxifying role of intracellular GSH, are of particular interest in the HIV-infected population where the majority of studies have demonstrated significant reductions in systemic and intracellular GSH levels in acquired immunodeficiency syndrome (AIDS) patients (Buhl *et al*, 1989; Staal *et al*, 1992; Aukrust *et al*, 1995; Herzenberg *et al*, 1997). Our data may provide a biochemical basis for the increased susceptibility of AIDS patients to sulfonamide-induced cutaneous reactions (Jaffe *et al*, 1983;

[3]Dietrich A, Kawakubo Y, Rzany B, Mockenhaupt M, Augustin M, Korner M, Wiek K, Stocker U, Baur S, Simon JC, Schopf E: Patients with severe cutaneous drug reactions (EM, SJS, TEN) have a reduced N-acetylating capacity. *J Invest Dermatol* 100:A519, 1993 (abstr.)

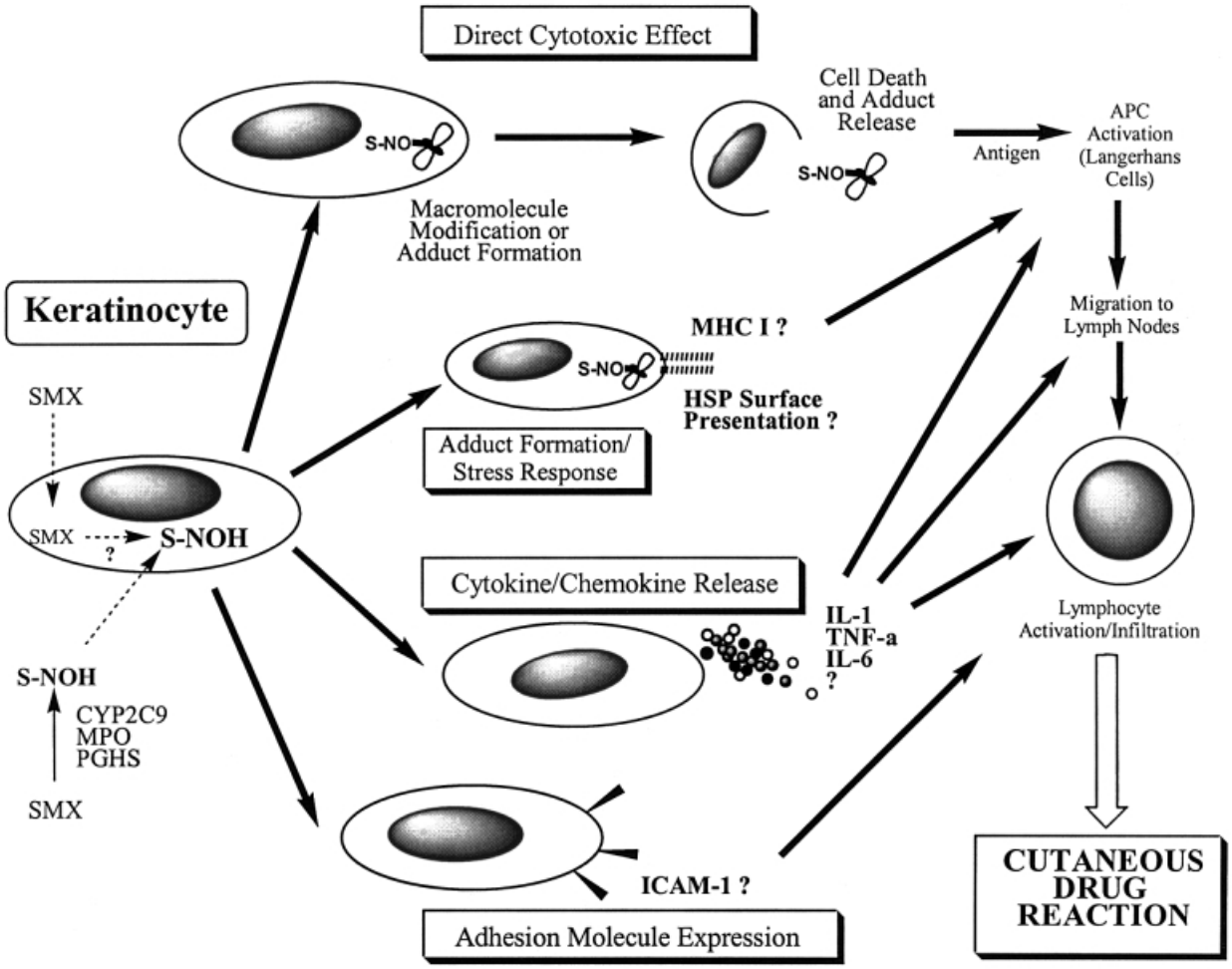

Figure 11. Proposed mechanism of sulfonamide-induced CDRs. Abbreviations: APC, antigen presenting cell(s); CYP2C9, cytochrome P450 2C9; HSP, heat shock protein(s); ICAM-1, intercellular adhesion molecule 1; IL, interleukin; MPO, myeloperoxidase; PGHS, prostaglandin H synthase (also known as COX); SMX, sulfamethoxazole; S-NOH, SMX-hydroxylamine; TNF-α, tumor necrosis factor α.

Gordin *et al*, 1984). Interestingly, one recent report also suggests that sulfonamide therapy itself may actually reduce intracellular GSH content in HIV-infected patients.[4]

Although the role of cytotoxicity in the development of sulfonamide-induced HS still remains to be determined, it has been proposed that the covalent interaction of reactive metabolites with cellular macromolecules is also a critical step in the manifestation of adverse effects (Pohl *et al*, 1988). The hapten hypothesis suggests that these interactions create neoantigens that can be recognized by the immune system as being ''nonself'' (according to traditional immunology dogma) or ''dangerous'' (according to the ''danger theory'' (Matzinger, 1998). Using ELISA and immunoblot analysis, we have demonstrated that SMX-NOH and DDS-NOH do covalently interact with cellular proteins of both normal human epidermal keratinocytes and PBMC. Cribb *et al* (1996b) had previously shown covalent binding of SMX-NOH to liver proteins that comigrated with binding targets of the reactive metabolite of halothane (Pohl *et al*, 1988). The relevance of these findings is still unknown in light of the fact that hepatotoxic manifestations of SMX-induced HS reactions are rare. The similarity of these observations, however, wherein prominent drug adducts are present at ~90–100 kDa and ~55 kDa in normal human epidermal keratinocytes, PBMC, and liver alike, lends credence to these being central targets of reactive metabolites and suggests some commonality among a variety of different ADR. Interestingly, binding was also increased with prior GSH depletion further supporting its critical role in preventing manifestations of drug toxicity. Further work will be required to identify which normal human epidermal keratinocyte proteins become selectively bound by hydroxylamine metabolites derived from exogenous *versus* intracellular sources, and whether this binding plays any role in the development of an immune-mediated response.

The fact that covalent adduct formation of SMX-NOH with normal human epidermal keratinocyte proteins occurred at concentrations where no toxicity was observed may indicate that covalent binding is unrelated to the cytotoxic effect of SMX-NOH. These data might also suggest the possibility that drug-protein adducts formed within viable normal human epidermal keratinocytes may be directly presented to dendritic epidermal T cells, possibly in the context of heat shock proteins or major histocompatibility complexes (MHC). Indeed, normal human epidermal keratinocytes have been shown to acquire antigen presenting cell (APC) function that is dependent, in part, on their expression of MHC class II antigen and ICAM-1 (Nickoloff *et al*, 1993; Nakano, 1998). Alternatively, these data might support the hypothesis that covalent adduct formation occurs to some extent in all normal human epidermal keratinocytes. At some ''threshold'' level of binding and presumed damage that may accumulate over the usual 7–14 d of drug therapy that precede an HS reaction, however, cells may die and release their contents into the extracellular environment only to be taken up by professional APCs (i.e., Langerhans cells) capable of initiating an immune

[4]Schiedel D, Tucker J, Wijsman J, Rieder M: Alterations of intracellular glutathione levels in PBMCs of HIV patients during sulfonamide therapy. *Clin Pharmacol Ther* 65:158: 1999 (abstr.).

response. This ''threshold'', which would be expected to vary between individuals, possibly as a function of detoxification ability, may be the primary determinant of who develops an HS response.

The aforementioned data suggest that normal human epidermal keratinocytes may indeed play an important role in the development of sulfonamide-induced cutaneous reactions. Taken together with the immunohistologic analysis of skin lesions taken from HS patients, as well as previous work with contact-sensitizing agents, we propose **Fig 11** as a working hypothesis for the mechanism of CDR to sulfonamides. This hypothesis suggests that reactive metabolites generated from local as well as systemic sources might (i) cause a direct cytotoxic effect; (ii) stimulate stress signal(s) that manifest as the surface expression of stress molecules and/or the release of cytokines; and (iii) stimulate the expression of adhesion molecules that promote lymphocyte infiltration and activation. These studies have shown that normal human epidermal keratinocytes can bioactivate SMX to a reactive hydroxylamine whose toxicity and covalent binding depend, at least in part, upon GSH depletion. Interestingly, SMX-NOH-induced GSH depletion and adduct formation can both occur in the absence of cell death, but where the cell may view itself as being under ''stressful'' conditions. Under different experimental conditions, ''stressed'' normal human epidermal keratinocytes show enhanced antigen processing and presentation (Cristau *et al*, 1994; Wells *et al*, 1998) and the ability to activate dendritic epidermal T cells directly (Havran *et al*, 1991; Huber *et al*, 1995). It remains to be determined if SMX-NOH might also induce such a stress response, either directly or indirectly through an alteration in cytokine release and/or adhesion molecule expression, with the end result being a cutaneous reaction.

Importantly, the proposed hypothesis might also provide a basis for the varied susceptibility of patients to drug-induced HS. Viral infection, for instance, might alter normal cytokine release within cutaneous tissues leading to the activation of previously dormant APCs. This APC activation would presumably enhance the likelihood that an immune response be initiated against neoantigens formed by the covalent binding of reactive metabolites to cellular macromolecules. Normal human epidermal keratinocytes under such stress might also exhibit elevated levels of inducible cyclooxygenase (COX-2) (Kanekura *et al*, 1998), which can catalyze the formation of reactive hydroxylamine metabolites (Goebel *et al*, 1999) and increase neoantigen levels within the local environment of the skin. These and related mechanisms may explain why certain subpopulations (e.g., HIV-infected individuals, patients with mononucleosis) appear to be predisposed to CDRs (Pullen *et al*, 1967; Carr and Cooper, 1995). Studies are ongoing in the authors' laboratory in an attempt to link cellular and subcellular events with the actual development of CDRs *in vivo*.

The authors acknowledge Dr. Alastair E. Cribb (Department of Anatomy and Physiology, Atlantic Veterinary College, PEI, Canada) for his generous gift of anti-SMX rabbit serum. We also thank Alan Davis and William Wurster (Department of Pharmaceutical Sciences, Wayne State University), as well as David Putt (Department of Pharmacology, Wayne State University) for their excellent technical assistance. T.P. Reilly was supported by a Pre-Doctoral Fellowship in Pharmacology/Toxicology from the Pharmaceutical Research and Manufacturers of America Foundation (PhRMA), Washington, DC. This work was supported in part by National Institutes of Health grant AI41395 to C.K. Svensson.

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